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Acidic solvent extraction of gossypol from cottonseed meal

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ABSTRACT

To expand the use of cottonseed protein in animal feeding, cottonseed meal was extracted with acetone- and ethanol-based solutions to remove gossypol. Phosphoric acid and water were included in the solutions to catalyze the hydrolysis of protein-bound gossypol. Both solvents were effective at reducing the total gossypol level in meal to between 5% and 10% of its initial value. Gossypol extraction occurred much faster in the ethanol-based extractions than it did in the acetone-based extractions. Treated meals tended to retain phosphorus but most of this could be removed by conducting a final water wash. Water washing also removed hydrophilic components resulting in reduced product yields but increased protein levels. Other acids, e.g., oxalic, citric, or sulfuric acid, were also effective at reducing meal gossypol. In contrast, extractions conducted without acid were not effective. The process can be used to produce low-gossypol cottonseed meals that should be useful in a broader range of feed applications.

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1. Introduction

Gossypol is a polyphenolic terpenoid that exists in the cotton plant as a defense agent and is known to be responsible for toxicity issues associated with the over feeding of cottonseed and cottonseed meal to animals (Berardi and Goldblatt, 1980). In addition to animal toxicity, the compound is studied for its anti-cancer, anti-viral, and male infertility effects (Wang et al., 2009). Ruminant animals tend to handle the effects of gossypol better than non-ruminant animals. Consequently, the meal is used almost entirely as a ruminant feed ingredient. Because of the presence of gossypol, the potential for expanding the use of cottonseed meal as a feed ingredient is limited.

Recent experiments have shown that cottonseed proteins derived from glandless varieties (named because they lack the glands that store gossypol and have only very low levels of the compound) can be substituted for much of the fish meal used in aquaculture diets (Siccardi et al., 2012). These experiments, conducted with meals, concentrates, and isolates prepared from glandless seed, were undertaken in the hope that recently reported RNAi-engineered cotton varieties (Sunilkumar et al., 2006), designed to eliminate gossypol only in the seed, might become commercial. Despite the potential advantages of these modified seeds, there are questions as to when these varieties will become available because of regulatory issues associated with planting a genetically modified organism and the intellectual property rights associated with their development. Hence, it may be some time for these varieties to become commercial.

This then suggests that it might be useful to consider the chemical removal of gossypol to produce meals that might be used favorably in other feeding applications, such as in aquaculture feeds. Because a substantial portion of the

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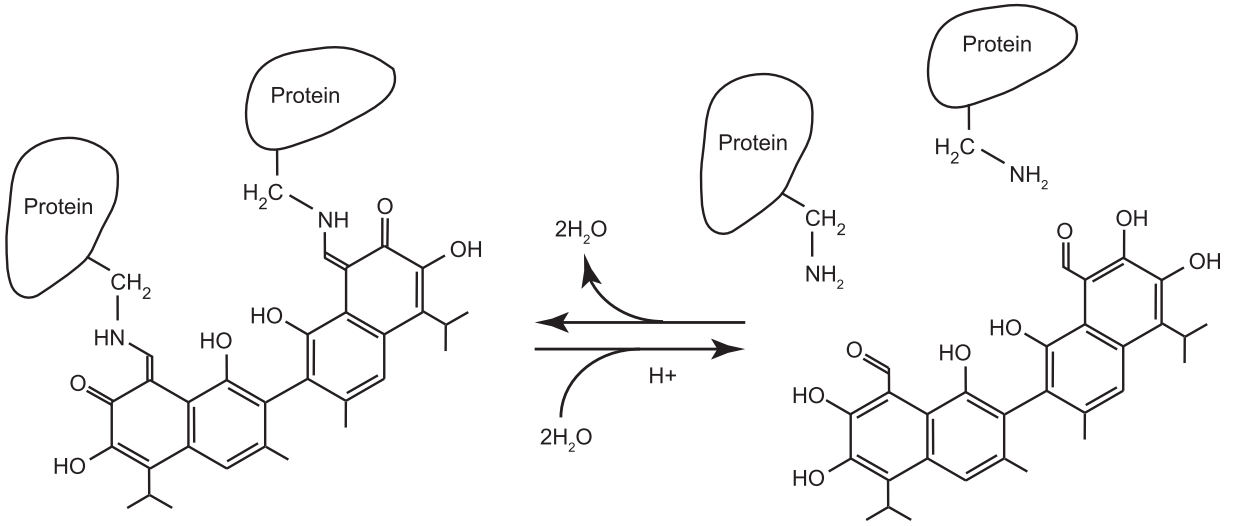

Fig. 1. Structure of gossypol and the reversible chemistry associated with the binding and hydrolysis of gossypol and protein.

gossypol present in cottonseed meal is bound to protein (as Schiff's bases formed with amino groups of lysine), the extractions need to be conducted under conditions that favor hydrolysis of bound gossypol (Fig. 1). Acid has been used to hydrolyze gossypol Schiff's bases formed with simple amines (Matlin et al., 1987; Dowd and Pelitire, 2006). To test for the possibility of extracting gossypol from cottonseed meal, commercial meal was extracted with solvent, water, and acid. The solvents used were acetone and ethanol, both GRAS (generally considered as safe) for use in food processing and capable of dissolving gossypol. The acid used was phosphoric acid, which is commonly used in vegetable oil processing. In addition, a few experiments were conducted with other solvents and acids to test the flexibility of conditions needed to achieve gossypol reduction.

2. Materials and methods

2.1. Materials

The meal used in the study was donated by Cotton Inc. (Cary, NC). The meal was made at the Food Protein Research Center (College Station, TX) primarily as a control material for aquaculture feeding experiments. It was produced under typical expander-solvent extraction conditions, except that the usual addition of hull material back to the kernels was limited to increase the meal's protein content.

Acetone, methanol, ethanol, phosphoric acid, and citric acid were purchased from Thermo-Fisher Scientific (Fair Lawn, NJ, USA). Oxalic acid was from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA) and sulfuric acid was from EM Sciences (Gibbstown, NJ, USA). All chemicals used were analytical grade or better.

2.2. Extractions

Twenty-five grams of meal was weighed into a 500-mL flat-bottom flask with 250 mL of an extraction solvent. Extraction solvents consisted of 80:20, 90:10, or 95:5 (v/v) acetone/water or 80:20, 90:10, or 95:5 (v/v) ethanol/water each with phosphoric acid added to give a 1.4 M solution. A stir bar was added to provide mixing. The flask was fitted with a condenser, and the mixtures were heated to reflux. Extraction times varied from 0.5 to 5.0 h for the acetone-based solutions and from 0.5 to 2.0 h for the ethanol-based solutions. After the extraction period, flasks were separated from the condensers and cooled to room temperature in an ice-water bath. Each sample was vacuum filtered over a Buchner funnel on #4 Whatman paper. After separation of the solvent, the retained meal was washed on the Buchner funnel to eliminate the gossypol contained in the hold-up volume. Two wash conditions were considered. For one set of experiments, the meal was washed with 250 mL of the same solution used for the extractions but without the acid. For the second set of experiments, the meal was first washed with 250 mL of the same solution used above but was then washed with an additional 250 mL of water. After the wash treatments, the meals were air dried under the hood to remove the bulk of the solvent and water and were then dried in a convection oven overnight at 50 °C. After cooling in a desiccator, each sample was weighed. Dry mass yields were determined from these weights and measured moisture levels (discussed below). A few extractions were also conducted with methanol, with the other acids listed above, or without the addition of acid.

2.3. Compositional analyses

The initial meal was ground through a Wiley mill fitted with a #20 screen before the extractions, and the extracted meal samples were re-ground through the same mill and screen before analyzing for composition. Both the initial and extracted meal samples were evaluated for moisture, protein, gossypol, crude oil, and phosphoric acid. These analyses were conducted in duplicate on each sample, and the results were averaged.

For moisture analysis, ~2.0 g samples were weighed into small pans and were dried at 130 °C in a convection oven for 2.0 h, as recommended in AOCS Official Method Ba 2a-38 (1998). The samples were then allowed to cool in a desiccator for 5 min, and moisture was determined by weight loss.

Gossypol was determined by a procedure slightly modified from AOCS Recommended Practice Ba 8a-99 (1998). Briefly, 100 mg of ground meal was mixed in a screw-capped test tube with 2.0 mL of a complexing reagent that consisted of 2:10:88 (v/v/v) *R*-(-)-2-amino-1-propanol (Sigma-Aldrich, Milwaukee, WI, USA), glacial acetic acid, and dimethylformamide. The test tubes were heated at 100 °C for 30 min in a dry bath to form a pair of diastereomeric Schiff's bases between gossypol and the chiral amine. After cooling, samples were diluted with 8 mL of HPLC mobile phase (described below) and were mixed. A portion of each sample was then transferred to a 2-mL microcentrifuge tube and centrifuged to remove particles. The clear supernatant was analyzed by HPLC.

Separation and detection of the gossypol complexes was achieved with a Waters (Milford, MA) model 2695 HPLC pumping system, model 996 photodiode array detector, and a reverse-phase SGE Inertsil column (ODS-2, 5 μm particle stationary phase, 100 × 4.6 mm). The mobile phase was 78% acetonitrile and 22% 10 mM potassium phosphate buffer (adjusted to pH 3.0 with phosphoric acid). Mobile phase was pumped at 1.0 mL/min. Injections were 20 μL, and gossypol was detected at 254 nm. Concentrations were determined by relating sample peak areas to standard curves prepared with racemic gossypol acetic acid (89.62% gossypol).

Protein content was determined by combustion. Each meal was weighed (~150 mg) into a tin foil and was analyzed for nitrogen on a Leco Truspec analyzer (Leco Corp, St. Joseph, MI). A conversion factor of 6.0 was used to convert nitrogen into protein, as this value was found to agree with values derived from reported cottonseed meal amino acid profiles (Dowd and Wakelyn, 2010).

Meal phosphoric acid levels were measured after silylation and detection of the resulting silyl phosphate ester by gas chromatography. Meal (50 mg) was weighted into a 5-mL reaction vial with 250 μL of pyridine, 500 μL of hexamethyldisilazane, 250 μL of a known solution of methyl-β-D-glucopyranoside in pyridine (as an internal standard), and 50 μL of trifluoroacetic acid. The mixture was heated at 60 °C for 45 min with periodic mixing and was then cooled. After allowing the solids to settle to the bottom of the vial, a 1-mL portion of the liquid phase was transferred to an autosampler vial for gas chromatography.

A model 7890A Agilent (Santa Clara, CA) gas chromatograph was used for detection of the esterified phosphate. The instrument was fitted with an Agilent/J&W Scientific DB-5HT (15 m × 0.25 mm i.d. × 0.1 μm film thickness) capillary column and was controlled with Chemstation software. The injector was operated in split mode with a split ratio of 1:100, and the injector and detector temperatures were set at 360 °C. The oven temperature profile was as follows: 120 °C for 3 min, ramped at 10 °C/min to 360 °C, and then held at 360 °C for 23 min to give a total run time of 50 min. The level of phosphoric acid was determined by internal standardization from the peak areas for the derivatized phosphoric acid and methyl β-D-glucopyranoside components, the known mass of methyl β-D-glucopyranoside in the preparation, and a response factor determined by analyzing a series of solutions of these compounds at known concentrations (Kaiser, 1977).

The oil content of the original meal was determined by extraction with a Foss North America (Eden Pairie, MN, USA) model HT6 Soxtec extraction unit. The instrument was operated with 3 g of meal, 40 mL of petroleum ether, a 15-min cook period, and a 2-h extraction period. The oil recovered was dried to a constant weight, and the oil content was determined by weighing.

For the initial meal and a few extracted meals with unexpectedly high mass yields (some above 100%), total phosphorus was determined by digestion of the meal in mineral acids followed by detection of the resulting phosphate by inductively coupled plasma-atomic emission spectroscopy. This test was performed by Galbraith Laboratories (Knoxville, TN, USA).

2.4. Replication and statistical analysis

Each set of extractions was conducted in triplicate with averages and standard deviations reported. An analysis of variance was conducted for a completely randomized design with a factorial arrangement of solvent-to-water ratio and extraction time. Multiple comparison tests were made by the least significance difference method ($\alpha = 0.05$). Yield, gossypol, protein, and phosphoric acid levels were included as dependent variables. Analyses were conducted on each solvent system (acetone and ethanol) and on the combination of solvent system and wash procedure (solvent washed or solvent and water washed). Correlation statistics were calculated among meal yield, protein, and phosphoric acid levels. In addition, regression analysis was performed on treatment means to explain the influence of time on gossypol. These relationships were compared between solvent water ratios and with and without water washing. This analysis was used only for the acetone extractions because limited time points were available for the ethanol extractions.

Table 1
Acetone extraction of cottonseed meal to remove gossypol.[a]

Solvent/water ratio	Time, h	Meal yield, g/kg	Gossypol[b], g/kg	Protein, g/kg	Phosphoric acid, g/kg
Solvent wash only					
80:20	0.5	946 ± 3	7.14 ± 0.12	536 ± 4	4.5 ± 0.5
	1.0	922 ± 3	5.77 ± 0.12	542 ± 7	4.8 ± 0.6
	2.0	901 ± 13	3.35 ± 0.03	543 ± 11	4.4 ± 0.5
	3.0	879 ± 10	2.19 ± 0.14	561 ± 3	4.0 ± 0.3
	5.0	870 ± 18	1.11 ± 0.02	568 ± 9	3.2 ± 1.3
90:10	0.5	1028 ± 8	6.74 ± 0.18	502.8	5.8 ± 0.6
	1.0	1019 ± 4	5.30 ± 0.16	505 ± 3	7.0 ± 0.7
	2.0	1005 ± 2	3.32 ± 0.04	508 ± 3	4.6 ± 0.6
	3.0	1002 ± 2	1.96 ± 0.03	513 ± 4	6.1 ± 0.5
	5.0	976 ± 17	1.01 ± 0.01	519 ± 9	6.8 ± 1.7
95:5	0.5	1093 ± 5	5.71 ± 0.23	469 ± 2	11.4 ± 0.9
	1.0	1086 ± 8	4.65 ± 0.09	473 ± 6	13.3 ± 0.1
	2.0	1071 ± 8	2.83 ± 0.09	477 ± 2	9.6 ± 0.9
	3.0	1064 ± 9	1.81 ± 0.02	482 ± 8	9.1 ± 1.8
	5.0	1050 ± 0	1.02 ± 0.01	488 ± 0	8.3 ± 0.9
LSD[a]		15	0.20	10	1.5
Solvent followed by water wash					
80:20	0.5	764 ± 16	10.29 ± 0.38	645 ± 10	1.3 ± 0.3
	1.0	733 ± 17	8.26 ± 0.14	664 ± 10	1.2 ± 0.2
	2.0	699 ± 15	4.27 ± 0.15	687 ± 9	1.1 ± 0.2
	3.0	694 ± 15	2.55 ± 0.06	686 ± 12	1.1 ± 0.3
	5.0	663 ± 4.5	1.26 ± 0.02	693 ± 26	1.0 ± 0.7
90:10	0.5	767 ± 14	9.96 ± 0.62	642 ± 11	1.3 ± 0.0
	1.0	750 ± 7	7.39 ± 0.26	656 ± 5	1.1 ± 0.3
	2.0	741 ± 6	4.36 ± 0.25	656 ± 7	1.7 ± 0.0
	3.0	700 ± 28	2.98 ± 0.18	683 ± 20	1.0 ± 0.4
	5.0	686 ± 42	1.49 ± 0.09	691 ± 34	0.9 ± 0.4
95:5	0.5	783 ± 23	8.59 ± 0.34	630 ± 14	0.9 ± 0.2
	1.0	773 ± 3	6.71 ± 0.13	632 ± 4	1.0 ± 0.1
	2.0	698 ± 14	4.52 ± 0.08	681 ± 15	1.0 ± 0.3
	3.0	696 ± 6	2.92 ± 0.18	687 ± 18	0.7 ± 0.2
	5.0	672 ± 10	1.53 ± 0.05	697 ± 8	0.6 ± 0.0
LSD		35	0.41	26	0.5

[a] LSD = least significant difference ($\alpha = 0.05$).
[b] Gossypol level (dwb) in initial meal = 11.7 g/kg.

3. Results

The moisture level in the initial cottonseed meal sample was 89 g/kg (as is basis). The meal was found to have 537 g of protein and 16 g of oil per kilogram of dry meal (dwb). The initial gossypol level in the meal was 11.7 g/kg (dwb), and 57% of the gossypol was in the (+)-optical form. Total meal phosphorus was 15.8 g/kg (dwb), and the level of phosphoric acid in the initial meal was 1.2 g/kg (dwb). The crude oil, gossypol, and phosphorus levels and the gossypol isomer ratio were typical of commercial cottonseed meals. The meal protein level was somewhat higher than is standard for commercial meal (~410 g/kg), which was expected because of the reduced addition of hulls back to the meats (i.e., kernel tissue) during oil extraction.

Extraction of the meal with either solvent in the presence of acid reduced total gossypol levels, and the reduction was progressive with time for each combination of solvent and wash treatment ($P < 0.0001$) (Tables 1 and 2). The acetone-based extractions required 5 h to reduce gossypol levels to ~10% of their initial values (Table 1). The rate of reduction for the acetone extractions (regression slope of log transformed data) indicated that the rate of decrease was significant ($P < 0.0001$) at about 65% per hour of extraction. Although water is required for gossypol hydrolysis, the rate of gossypol reduction was not significantly affected by the amount of water present in the system during extraction ($P = 0.196$).

For the ethanol extractions, it required only 0.5–1.0 h to reduce the gossypol level to levels comparable to the 5 h acetone extractions (Table 2). Because the rate of gossypol removal was faster in ethanol, the 3 and 5 h time points were not evaluated and there was an insufficient range of data to conduct similar rate analyses on these experiments. The distribution of the individual gossypol isomers in the residual meal was unchanged by the extractions in both sets of solvent experiments (data not shown).

The amount of water present during the extraction affected the yield of meal for both the acetone and ethanol extractions. Increasing the amount of water in the extraction solvent reduced yields (Tables 1 and 2). This was predictable, as increased co-extraction of hydrophilic components would be expected as the amount of water level in the solvent increased. The effect

Table 2
Ethanol extraction of cottonseed meal to remove gossypol.[a]

Solvent/water ratio	Time, h	Dry matter yield, g/kg	Gossypol[b], g/kg	Protein, g/kg	Phosphoric acid, g/kg
Solvent wash only					
80:20	0.5	783 ± 18	1.21 ± 0.05	589 ± 11	2.2 ± 0.3
	1.0	746 ± 1	0.86 ± 0.04	595 ± 5	1.7 ± 0.2
	2.0	735 ± 4	0.82 ± 0.02	588 ± 4	1.9 ± 0.1
90:10	0.5	909 ± 2	1.10 ± 0.02	537 ± 2	3.5 ± 0.1
	1.0	891 ± 4	0.75 ± 0.01	544 ± 5	3.7 ± 0.6
	2.0	876 ± 5	0.67 ± 0.01	549 ± 1	3.7 ± 0.1
95:5	0.5	957 ± 2	1.25 ± 0.00	514 ± 1	2.4 ± 0.2
	1.0	940 ± 4	0.73 ± 0.01	523 ± 2	4.0 ± 0.2
	2.0	917 ± 6	0.64 ± 0.01	525 ± 2	4.4 ± 0.8
LSD[a]		13	0.06	8	0.6
Solvent followed by water wash					
80:20	0.5	662 ± 21	2.02 ± 0.56	688 ± 11	0.7 ± 0.3
	1.0	614 ± 10	1.08 ± 0.03	695 ± 2	0.8 ± 0.3
	2.0	606 ± 16	1.24 ± 0.48	680 ± 23	1.0 ± 0.7
90:10	0.5	704 ± 4	1.44 ± 0.02	675 ± 6	0.7 ± 0.5
	1.0	677 ± 6	0.90 ± 0.04	689 ± 3	0.5 ± 0.1
	2.0	651 ± 13	0.73 ± 0.02	696 ± 6	0.5 ± 0.1
95:5	0.5	726 ± 10	1.71 ± 0.07	662 ± 6	0.3 ± 0.0
	1.0	696 ± 15	0.96 ± 0.02	675 ± 14	0.3 ± 0.1
	2.0	683 ± 8	0.76 ± 0.03	685 ± 12	0.3 ± 0.1
LSD		22	0.42	19	0.6

[a] LSD = least significant difference ($\alpha = 0.05$).
[b] Gossypol level (dwb) in initial meal = 11.7 g/kg.

was especially pronounced (highest *F*-values) when the final meal product was only washed with the solvent solution used for extraction ($P < 0.0001$ for both series). When the meal was also given a final wash with water, product yields were less strongly influenced by the amount of water included during extraction. In effect, the water wash removed the hydrophilic components that were being partially extracted by the solvent/water solutions. The water level in the solvent was significant ($P < 0.0001$) for the ethanol-based series but not significant for acetone-based series ($P = 0.07$).

For the 90:10 and 95:5 (v/v) acetone–water extractions, the yield of extracted meal was greater than the initial meal weight when the product was only washed with acetone/water (Table 1). Although the 80:20 (v/v) acetone–water extraction yields were reduced, they were still greater than the yields observed for the equivalent ethanol-based extractions (Table 2). In addition to reduced extraction of polar components when less water was present in the solvent, the elevated yields appeared to result from the retention of phosphoric acid after extraction. Analysis of phosphoric acid levels showed that the meals with higher yields contained more residual phosphoric acid (Pearson-*R* value = 0.9014, $P < 0.0001$). The levels, however, were not high enough to account for most of the excess yield, suggesting that some phosphoric acid might be bound to meal components during extraction.

To test if phosphorus was being retained by the meals, four samples were analyzed for total phosphorus. The initial meal contained 17 g/kg (dwb) phosphorus, which is a little above typical cottonseed meal levels of around 10 g/kg phosphorus (Cherry and Leffler, 1984). Refluxing the meal with 95:5 (v/v) acetone/water and 1.4 M phosphoric acid for 2 h and washing only with the solvent mixture increased the total phosphorus level in the meal to 92 g/kg (dwb). Assuming that this phosphorus is in the form of phosphate, this increase would more than account for the observed excess yield (Table 1). Adding a water wash after the solvent wash reduced the total phosphorus level of this meal to 36 g/kg (dwb). Extracting the meal with 95:5 (v/v) ethanol/water plus 1.4 M phosphoric acid for 2 h and washing the meal only with the solvent mixture minus the acid also produced residual meal with an increased phosphorus level of 81 g/kg, indicating that the same phosphorus retention was occurring in these extractions despite the yields being less than 1000 g/kg (dwb).

Regardless of the solvent used, meal protein levels were influenced by the water level in the extractions ($P < 0.0001$) and were even more strongly influenced (greater *F*-value) by the addition of a final water wash after the extractions ($P < 0.0001$). Over all the experiments, meal protein level correlated inversely with meal yield (Pearson-*R* value = –0.9726, $P < 0.0001$). Water washing increased meal protein levels to those close to protein concentrates (i.e., around 70%) in both the acetone and ethanol extractions.

Extractions conducted without acid removed only modest levels of gossypol (Table 3). Gossypol levels slightly increased in these samples because of the greater removal of other components. Extractions conducted with oxalic or citric acids at the same concentration were similarly effective in reducing gossypol levels (Table 3). Extractions conducted with sulfuric acid were also effective but with this stronger mineral acid lower acid levels were needed to prevent significant degradation

Table 3
Miscellaneous solvent extraction of cottonseed meal to remove gossypol.[a]

Extraction conditions:			Dry matter yield, g/kg	Total gossypol[b], g/kg	Protein, g/kg
Solvent, acid level, and washes	Solvent/water (v/v) ratio	Time, h			
Acetone, no acid, solvent and water washed	95:5	5.0	792 ± 6^{A}	12.65 ± 0.18^{C}	671 ± 4^{E}
Methanol, no acid, solvent and water washed	95:5	2.0	760 ± 6^{B}	13.77 ± 0.33^{A}	692 ± 6^{D}
Methanol, no acid, solvent and water washed	95:5	5.0	767 ± 3^{B}	13.31 ± 0.21^{B}	693 ± 3^{D}
Ethanol, no acid, solvent and water washed	95:5	2.0	766 ± 6^{B}	14.02 ± 0.15^{A}	694 ± 7^{D}
Ethanol, 1.4 M acetic acid, solvent and water washed	95:5	2.0	769 ± 6^{B}	12.20 ± 0.42^{D}	695 ± 2^{D}
Ethanol, 1.4 M citric acid, solvent and water washed	95:5	2.0	684 ± 8^{C}	1.48 ± 0.06^{E}	712 ± 8^{C}
Ethanol, 1.4 M oxalic acid, solvent and water washed	95:5	2.0	663 ± 5^{D}	0.81 ± 0.02^{F}	728 ± 6^{B}
Ethanol, 1.0 M sulfuric acid, solvent and water washed	95:5	2.0	473 ± 14^{G}	0.50 ± 0.02^{F}	757 ± 18^{A}
Ethanol, 0.50 M sulfuric acid, solvent and water washed	95:5	2.0	592 ± 1^{F}	0.78 ± 0.00^{F}	766 ± 6^{A}
Ethanol, 0.25 M sulfuric acid, solvent and water washed	95:5	2.0	646 ± 4^{E}	0.82 ± 0.04^{F}	761 ± 5^{A}
LSD			11	0.34	13

[a] Values within a column are significantly different if superscripted with a different letter. LSD = least significant difference ($\alpha = 0.05$).
[b] Gossypol level (dwb) in initial meal = 11.7 g/kg.

of the meal. Even with 0.25 M sulfuric acid, additional yield loss was apparent (Table 3) and the treated meal had a notably darker appearance.

4. Discussion

Extraction of cottonseed meal with solvent and acid was effective at reducing gossypol levels by 90–95%. Each solvent was effective, although the extraction was faster in ethanol compared with acetone. That extractions conducted without acid did not reduce meal gossypol levels, indicating that most of the gossypol was present in a bound form. These meals had higher gossypol levels than the starting material because of greater removal of other components (Table 3).

Phosphoric acid was used for this work because the oilseed industry uses it to separate non-hydratable phospholipids from crude oils and to reduce metal levels in refined oils. The level of phosphoric acid used in this work was based on prior reports that focused on the preparation of gossypol from cottonseed "gums", i.e., hydratable phospholipids (Pons et al., 1959), and soapstock (Dowd and Pelitire, 2001). From these studies, 1.4 M phosphoric acid maximized the recovery of gossypol when the gums or soapstock were refluxed in methyl ethyl ketone for 2 h. Similarly, 1.4 M phosphoric acid was also used to follow the rate of hydrolysis of the di-3-amino-1-propanol-gossypol adduct, which was used to effect the separation of methylated gossypol Schiff's bases by chromatography (Dowd and Pelitire, 2006).

This choice of acid, however, resulted in an unintended consequence in that significant amounts of phosphorus were retained by the meals, resulting in dry matter yields being greater than the initial weight of the meal for a few of the treatments (Table 1). Initially, we thought that this occurred because of partitioning of some phosphoric acid with the meal during the separation of the extraction solvent. This was suggested because the excess yield was associated with the extractions conducted with higher concentrations of acetone (a less polar solvent environment compared with ethanol) and because water washing eliminated the extra mass (Table 1). Analysis of meal phosphoric acid levels did suggest such an effect (Table 1) but the levels found (<14 g/kg) were not large enough to account for the bulk of the excess mass yield. Phosphorus analysis of a few samples indicated that phosphate retention did account for the excess mass. As this was not measurable as phosphoric acid, it suggests that some covalent binding of the acid was occurring. Because cottonseed meal has considerable amounts of oligosaccharides, polysaccharide gums, and cellulosic components (Kuo et al., 1988; Zarins and Marshall, 1988), there are many hydroxyl groups available for phosphate esterification. In the acetone-based experiments, much of this carbohydrate fraction and any bound phosphate would be retained within the meal. In the aqueous ethanol extractions, some but not all of the carbohydrate components will be extracted, and if the unextracted fraction contains bound phosphate, it would be retained.

The presence of additional phosphorus in the meal may not be undesired if the meal is to be used in aquaculture diets. As phosphate levels in fresh and sea waters are low, phosphorus is a dietary requirement for fish produced by aquaculture (National Research Council, 1993), and phosphate salts are often added to fish meals (Åsgård and Shearer, 1997). Consequently, the elevated levels of phosphate in these materials may have some advantages for this application, although the

availability of this phosphate in fish diets will need to be assessed. Because bypass loss of phosphorus is an environmental concern, phosphate requirements may need to be re-evaluated if these meals are included in fish rations. Alternatively, other acids appear just as capable of hydrolyzing gossypol Schiff's bases and lowering total gossypol levels (Table 3), and retained phosphorus would not be of concern if these acids were used. In the present study, oxalic, citric, and sulfuric acids were effective at reducing the total gossypol level of cottonseed meals. Acetic acid was too weak to be effective at a similar concentration.

Water washing the extracted meals resulted in the removal of some meal components and reduced product yields by a considerable amount. However, it also increased meal protein levels to those close to protein concentrates. Hence, the water wash removed significant amounts of hydrophilic components, most likely including non-cellulosic carbohydrates, and it appeared to remove only minimal amounts of protein. As high protein levels in fish meals are desirable, it may be beneficial to trade some meal yield for the increased protein levels afforded by these treatments.

Extractions conducted without acid were ineffective at removing gossypol, which differs somewhat from the prior report by Saxena et al. (2012) that noted a 60% gossypol reduction by extraction with ethanol. This difference occurs because the meal used in this prior work was carefully defatted at low temperature to avoid the binding of gossypol to meal components. This situation is somewhat artificial, as commercial oil extraction requires that the seed undergo preparatory steps and many of these operations (e.g., roller milling to form flakes, cooking to aggregate the protein and coalesce the microscopic oil droplets, and extrusion to form a solvent-extractable porous pellet) promote the binding of gossypol to the meal's protein fraction. These steps are important not only to effect oil extraction but the gossypol binding that occurs is desired to reduce the amount of the compound co-extracted with the oil, as it can cause problems during oil bleaching.

In this work, extraction in ethanol removed gossypol more quickly than did extraction with acetone. This difference appears to result from the difference in the reflux temperatures, i.e., 56–58 °C for the acetone-based extractions and 82–83 °C for the ethanol-based extractions, although a contribution by the more protic ethanol cannot be excluded. This also suggests that extractions conducted in even higher boiling solvents, e.g., 2-propanol, might remove gossypol more rapidly. There is a tradeoff, however, between the speed of the extraction and in the costs associated with the process, as the energy needed to conduct the extractions and recover the solvent increases with higher boiling materials. In addition, meal desolventization may need to be more carefully controlled if the solvent used is not GRAS for food or feed processing, which may add further to costs.

Extraction of gossypol from cottonseed products is not a new idea, and a number of approaches have been proposed. Many studies have focused on reducing the so-called "free" gossypol level in cottonseed meal. Various physical methods (e.g., Zhuge et al., 1988; Gerasimidis et al., 2007), chemical treatments (Hron et al., 1996; Liu et al., 1981), and biological approaches (e.g., Khalaf and Meleigy, 2008; Zhang et al., 2007) have all been utilized to promote the binding of gossypol to the meal protein. Generally, these reports claim "detoxified" or "safe" meals, often noting the WHO and FDA standards regarding safe levels of free gossypol for human consumption but without much animal testing to confirm or support the claims. Gossypol toxicity is a complicated subject, and many studies on the topic have yielded inconsistent and inconclusive results. The lack of a clear picture on this issue suggests that gossypol toxicity probably depends on many factors that include physiological conditions of the animals, environmental conditions, species differences, inherent animal-to-animal variation, the influence of other dietary components, in addition to the level and forms of gossypol present in the diet. Because the stomachs of monogastric animals have low pH environments, they provide conditions that favor the hydrolysis of bound gossypol. Hence, removal of only the "free" gossypol fraction does not guarantee that these products will be safe for non-ruminant animal feeding. Consequently, removal of total gossypol is preferable.

Fewer efforts have attempted to eliminate total gossypol from cottonseed products. Liadakis et al. (1993) have reported on the extraction of cottonseed meal with alcohols and modest amounts of hydrochloric acid (0.005 N initially, then adjusted to pH 4.5 during the extraction), similar to the approach used here. Their conditions, however, removed only a fraction of the gossypol (22–40%), probably corresponding to the meal's unbound gossypol fraction. This modest removal occurs because the acid level, extraction temperature (25 °C), and extraction time (30 min) were all insufficient to achieve more complete hydrolysis of the bound gossypol.

Among other reported approaches to reduce or eliminate total gossypol, the liquid-cyclone process has received the most attention. This process gently grinds kernels to keep the pigment glands intact then separates the glands from the other meal components with a cyclone (Vix et al., 1971). The process spawned some initial commercial interest but proved to be very difficult to engineer at scale. Damaty and Hudson (1975) proposed a multi-step extraction process, first extracting the oil with hexane, then rupturing the pigment glands by the addition of aqueous acetone, followed by gossypol extraction with anhydrous acetone. Meal total gossypol levels of 1 g/kg were reported by this method. For this approach to be effective, however, the seed must be flaked carefully at low temperature to reduce gland rupture and dried prior to and after hexane extraction conditions, as mentioned above, that are not optimal for oil recovery and add considerably to extraction cost. Hron et al. (1992) focused on extracting gossypol with the oil from full fat kernels using water-miscible solvents (but worked mostly with 95% ethanol) that contained a small amount of acid to limit the binding of gossypol. This process produced meal fractions with between 0.3 and 4 g/kg of total gossypol, compared with 14.8 g/kg obtained by a more traditional hexane extraction. The process, however, did not consider the complications involved with recovery of the oil from the gossypol-laden solvent or the complications that would result in refining this oil for color. Kuk et al. (1991) touched on the latter

issue by proposing the use of pervaporation to recover the solvent followed by the addition of an absorbent to separate gossypol from the oil. This approach, however, also adds significantly to cost and would still likely result in oil bleaching problems.

The process proposed here is simpler in that it would not interfere with the well established oil extraction process. It would still require, however, an additional solvent extraction and the use of acid to hydrolyze gossypol present in a bound form. The recovered meal would need desolventization to reduce solvent levels to acceptable limits and to maximize solvent recovery. The energy needed for this might be greater or lesser than needed for the recovery of hexane from defatted meal, as the heat of vaporization would be greater for more polar solvents than it is for hexane but the acceptable residual solvent levels in the meal would likely be higher than they would be for hexane. The use of the extracted meal solids is also a concern. Gossypol could be recovered from this byproduct material, and the remaining components (largely carbohydrates) could be used to support fermentations or could be added back to the degossypolized meal or to another untreated meal fraction. While all of these operations would add cost, the increased value of the meal as a partial replacement for fish meal might justify the additional processing.

5. Conclusions

Extraction of cottonseed meal with acetone or ethanol in the presence of phosphoric acid reduced total gossypol levels by 90–95%. The treatment also extracted some carbohydrate components, which resulted in greater concentrations of the meal protein. The removal of gossypol and carbohydrates and the increased protein should be beneficial for allowing the meal to be used as a more valuable partial replacement for fish meal in aquaculture feeds.

Conflict of interest

The authors confirm that there are no conflicts of interest or personal financial interests associated with the outcome of this work.

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